3. MATERIALS AND METHODS

Materials and methods followed in the present study were as follows:

EXPERIMENTAL DESIGN:

PHASE I
1. Plant collection and authentication.
2. Anatomical study of *Barleria cristata* L. leaves.
3. Phytochemical screening of *Barleria cristata* L. and *Rubia cordifolia* Linn.
5. Quantification of phytoconstituents and vitamins.

PHASE II
1. HPTLC analysis of *Barleria cristata* L. crude leaf extract for saponins profile.
2. HPLC analysis of *Barleria cristata* L. leaf extract for saponin identification using Bacopa (Bhrami) tablets as standard.
3. Assaying free radical scavenging ability of *Barleria cristata* L. and *Rubia cordifolia* Linn.
4. Determination of *in vitro* antioxidant potential of *Barleria cristata* L. and *Rubia cordifolia* Linn.

PHASE III
1. Preparation of 50% hydroethanolic extract of *Barleria cristata* L. and *Rubia cordifolia* Linn.
2. Acute toxicity study of extracts.
3. Hypoglycemic and Hypolipidemic activity of 50% hydroethanolic extract of *Barleria cristata* L. leaf extract.
4. Antidiabetic study of 50% hydroethanolic extract of *Barleria cristata* L. stem extract.
5. Antidiabetic study of *Rubia cordifolia* Linn. (root) and effect of combined *Barleria cristata* L. (stem and leaf) and *Barleria cristata* L. (stem) and *Rubia cordifolia* Linn. (root) extract in alloxan induced diabetic, hyperlipidemic rats.

Biochemical Analysis:

- Biochemical estimations in liver, kidney and serum.
- Estimation of enzymic antioxidants.
- Estimation of non – enzymic antioxidants.

**PHASE IV**

1. Antimicrobial activity of saponin fractions of *Barleria cristata* L. (leaf) and *Rubia cordifolia* Linn. (root).

2. Histological investigations of liver, kidney, pancreas tissue samples.

**PHASE I**

3.1 PLANT COLLECTION AND AUTHENTICATION

The plant *Barleria cristata* L. commonly known as Kodilkannu (in Southern India) was collected from the rural area around Erode district, Tamil Nadu in the month of December. A complete plant with flowers of *Barleria cristata* L. was identified by the Botanical Survey of India (BSI), Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India (N.BSI/SC/5/23/08-09/Tech.175).

The dried roots of *Rubia cordifolia* Linn. was purchased from a local well known herbal materials supplier of Tirupur, Tamil Nadu. A Voucher Specimen of the plant was collected from the lower hills area of Ooty and was authenticated by Dr. S. RAJAN, Department of AYUSH, Ministry of Health and Family Welfare, Govt. of India, Nilgiri District.
3.2. ANATOMICAL STUDY OF *Barleria cristata* L. LEAF

3.2.1. COLLECTION OF SPECIMENS

The plant specimens for anatomical studies were collected with care by selecting healthy leaf and normal organs. The fresh plant leaves were used. The required samples of different organs were cut and removed from the plant and fixed in FFA (formalin (5ml) + Acetic acid (5ml) + 70% Ethyl Alcohol (90 ml)]. After 24 hrs of fixing, the specimens were dehydrated with series of graded alcohol solutions as follows:

Reagents: Appendix I

3.2.2. INFILTRATION WITH PARAFFIN (Sass, 1940)

The material fixed in FAA or in any reagent was washed thoroughly in distilled water and the water was removed by the following process.

1. Washed with 70% ethyl or isopropyl alcohol – 1 day
2. Washed with 80% ethyl or isopropyl alcohol – 1 day
3. Washed with 100% ethyl or isopropyl alcohol – 1 day (Added a grain of eosin to colour the material)
4. Added xylol (about 15 drops) for every 10 minutes till alcohol and xylol become equal in volume (The specimen tubes were shaken gently to facilitate mixing of the chemicals)
5. Initial volume of alcohol was noted and marked
6. After xylol and alcohol become equal in volume, a part of the mixture was poured out
   The plant material was transferred to pure xylol and left for 1 day
7. The volume was made upto intial volume by adding xylol drops and kept for one day
8. Xylol was changed again after 1 day
9. Added thin shavings of wax – one pinch every 30 minutes till the xylol became supersaturated
Kept the specimen tube closed with cork in the thermostat at 40°C

Added some more wax and kept in the thermostat

The materials were transferred to pure molten wax after the super saturation was reached and the tubes were kept open in the thermostat at 55 – 58°C.

The material was changed in pure molten wax for 2 times at 6 hrs intervals

A block was prepared with the embedded material after 1 or 2 days (If ethyl alcohol was not available, isopropyl alcohol may be used instead of ethyl alcohol)

3.2.3. SECTIONING (Johansen, 1940)

The paraffin embedded specimens were sectioned with the help of Rotary microtome. The thickness of the sections were 10 – 12 µm. Then the sections were dewaxed and stained with toludine as follows.

3.2.4. TOLUDINE BLUE – STAINING (O’ Brien et al., 1964)

Added xylol and kept for 10 – 15 minutes

Added xylol and alcohol and kept for 5 minutes
Washed with 100, 90 and 70% alcohol

The slides were stained using toludine blue (the colour differentiation was noted)

Rinsed with distilled water to remove the excess stain

Again washed with 100, 90, 70% alcohol and xylol + alcohol

Dipped in xylol for 2 – 3 minutes and mounted with DPX
For studying stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) 5% sodium hydroxide and Jeffrey’s maceration fluid were prepared.

3.2.5. PHOTOMICROGRAPHS

Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used. Magnifications of the figures were indicated by the scale – bar.

3.3. PHYTOCHEMICAL SCREENING OF PLANT SAMPLES

The shade dried powdered plant material was subjected to soxhelet extraction with different solvents such as ethanol, acetone, chloroform, distilled water, petroleum ether and benzene for 18 hours. The extracts were condensed by rotary evaporator and were used for screening of phytochemicals.

3.3.1. QUALITATIVE ANALYSIS OF PHYTOCHEMICALS (Peach and Tracy, 1955 and Kokate et al., 2007)

Reagents: Appendix II

3.3.1. IDENTIFICATION OF CARBOHYDRATES
a. Molisch’s test

In a test tube containing 2 ml of the test solution, added a few drops of 20% alcoholic α–naphthol (2–naphthol) followed by few drops of concentrated sulphuric acid added through sides of the test tube. Purple to violet colour ring appears at the junction and its disappearance on the addition of aqueous sodium hydroxide solution indicated the presence of carbohydrates.
b. **Fehling’s test**

To 2 ml of the plant extract, 1 ml of mixture of equal parts of Fehling’s solution A and B was added. The contents were boiled for few minutes. Formation of red or brick red precipitate indicated the presence of carbohydrates.

c. **Benedict’s Test**

To 0.5 ml of the plant extract, 0.5 ml of Benedict’s reagent was added and boiled for 5 minutes. Formation of bluish – green colour showed the presence of carbohydrates.

### 3.3.1.2. IDENTIFICATION OF PROTEINS

a. **Millon’s test**

To 1 ml of the test solution, added 3 ml of water and 1 ml of Millon’s reagent, heated in a boiling water bath for few minutes and the contents were cooled. A white precipitate which turns red on heating indicated the presence of proteins.

b. **Biuret’s test**

To 1 ml of extract, 5 – 8 drops of 10% sodium hydroxide solution, followed by two drops of 3% copper sulphate was added. Formation of red or violet colour confirmed the presence of proteins.

### 3.3.1.3. IDENTIFICATION OF ALKALOIDS, FLAVONOIDS AND SAPONINS

#### ALKALOIDS

a. **Dragendroff’s test (Krout reagent – Potassium bismuth iodides)**

To 0.5 ml of test solution, added 2 ml of HCl. To this acidic medium, 1 ml of reagent was added. An orange, red precipitate produced immediately indicated the presence of alkaloids.

b. **Wagner’s test (Iodine – Potassium iodide solution)**

To 1 ml of test solution, added 1.55% v/v of HCl and few drops of Wagner’s reagent. Formation of yellow or brown precipitate showed the presence of alkaloids.
c. Mayor’s test (**Potassium mercuric iodide**)

To 1 ml of the plant extract few drops of Mayer’s reagent was added. Formation of white or pale yellow precipitate indicated the presence of alkaloids.

**FLAVONOIDS**

a. Shinoda test

To the test solution, added few magnesium turnings and concentrated HCl drop wise. Pink, scarlet, crimson red or occasionally green to blue colour appears after few minutes. This showed the presence of flavonoids.

b. Alkaline reagent test

To the test solution, added few drops of NaOH solution. Intense yellow colour was formed which turned colourless on addition of few drops of dilute HCl which indicated the presence of flavonoids.

c. Zinc Hydrochloride test

To the test solution, added a mixture of zinc and concentrated hydrochloric acid. It gave red colour after few minutes confirmed the presence of flavonoids.

**SAPONINS**

a. Foam test

In a test tube containing about 5 ml of test solution a drop of sodium bicarbonate solution was added. The mixture was shaken vigorously and kept for 5 minutes. A honey comb like froth was formed and it showed the presence of saponins.

b. Haemolysis test

A suspension of Red Blood Corpuscles (RBC) in normal saline was treated with a small amount of plant decoction, in the presence of saponin, hemolysis was observed.
3.3.1.4. IDENTIFICATION OF PHENOL, GLYCOSIDES, STEROIDS

PHENOLS

a. Ferric Chloride test

To 1 ml of the extract, 2 ml of distilled water followed by few drops of 10% aqueous ferric chloride solution were added. Formation of blue or green or violet colour indicates the presence of phenols.

b. Lead Acetate test

1 ml of test solution was diluted to 3 ml with distilled water and to this few drops of 1% aqueous solution of lead acetate were added. A yellow precipitate formed indicated the presence of phenols.

c. Libermann’s test

A small quantity of extract was dissolved in 0.5 ml of 20% sulphuric acid solution, followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution.

GLYCOSIDES

Keller- Killiani’s test

A small amount of the plant extract was dissolved in 1 ml of water and then aqueous sodium hydroxide solution was added. Formation of yellow colour indicated the presence of glycosides.

STEROIDS

a. Libermann – Burchard’s test

To 1 ml of the extract, 1 ml of concentrated sulphuric acid was added followed by the addition of 2 ml of acetic anhydride solution. A greenish colour developed and it turned to blue indicated the presence of steroids.
b. Salkowski reaction

To 2 ml of the extract, 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. A red colour produced in the chloroform layer confirmed the presence of steroids.

3.3.1.5. IDENTIFICATION OF RESINS, TANNINS, THIOLS

RESINS

To 2 ml of alcoholic extract 5 - 10 ml of acetic anhydride was added, dissolved by gently heating, cooled and then 0.5 ml of sulphuric acid was added. A bright purple colour rapidly changing into violet was produced indicated the presence of resins.

TANNINS

a. Ferric Chloride test

To 2 ml of extract, a few drops of 5% aqueous ferric chloride solution and few ml of dilute sulphuric acid were added followed by the formation of yellowish brown precipitate showed the presence of tannins.

b. Lead Acetate test

In a test tube containing 5 ml of extract few drops of 1% solution of lead acetate was added. A yellow or red precipitate formed indicated the presence of tannins.

THIOLS

To about 5 ml of plant extract, enough ammonium sulphate was added to saturate the solution, 2 – 4 drops of 5% sodium nitroprusside was then added followed by one or more drops of concentrated nitric acid. A transient magenta colour developed in presence of thiols.
3.4. DETERMINATION OF PROXIMATE COMPOSITION of *Barleria cristata* L. LEAF AND *Rubia cordifolia* LINN. ROOT POWDER.

For the determination of proximate composition, crude leaf powder was used.

**Reagent: Appendix III**

3.4.1. Determination of Moisture Content (African Pharmacopoeia, 1986)

The powdered plant material (2 g) was weighed into a clean crucible of known weight. After oven drying at 105°C for 5 hours the crucible was cooled and weighed to determine weight loss in the powdered drug. The average percentage weight loss with reference to the air dried powdered material was determined.

3.4.2. Total Ash Determination (Pharmacopoeia of India, 1996)

About 2-3 g of powdered plant material was incinerated in a silica dish at a temperature not exceeding 450°C until free from carbon. It was then cooled and weighed. The % w/w of ash with reference to the air dried plant material was calculated.

3.4.3. Determination of Ethanol Soluble Extractive (African Pharmacopoeia, 1986)

Accurately weighed 5 g of air dried powdered plant material was macerated with 100 ml of ethanol of the specified strength (90%) in a closed flask for 24 hrs, shaking frequently during first 6 hrs and allowed to stand for 18 hrs. It was then filtered rapidly, taking precautions against the loss of solvent and 25 ml of the filtrate was evaporated to dryness in a flat bottomed shallow dish and dried at 100°C to constant weight. The % w/w of ethanol soluble extractive value was calculated with reference to air – dried plant material.


Procedure was same as alcohol soluble extractive using chloroform and water (chloroform: water – 1: 399 v/v) instead of ethanol.

The crucible with the total ash experiment was transferred into a beaker containing 25 ml of distilled water. The beaker and its contents were boiled for 5 min and filtered through an ashless filter paper.

The filter paper containing the residue was folded and placed in a weighed porcelain crucible. The crucible was then heated in the muffle furnace, until the filter paper was completely ashed. The crucible and its content were cooled and weighed and the final weight noted. The weight of the residue was then calculated by subtracting the constant weight of the second crucible and its ash. This is the water insoluble ash. The weight of the water soluble ash was obtained by subtracting the weight of the water insoluble ash from the total ash. The weight of the water soluble ash divided by the initial weight of the crude drug was multiplied by 100 and was taken as the water soluble ash value.


The crucible with the total ash was transferred into a beaker containing 25 ml of diluted HCl. The beaker and its contents were boiled for 5 min and the boiled contents filtered through an ashless filter paper. The washings were then passed through the filter paper in a manner as to allow the collection of the residue at the tip of the cone of the filter paper. The weight of the clean and heated porcelain crucible was accurately determined. The filter paper with the residue was folded with a small cone and transferred into the crucible. The crucible was gently heated until the filter paper was completely ashed and heated strongly for a few minutes.

The crucible and its contents were cooled, weighed and the final weight was noted. The weight of the residue was then calculated. This was done by subtracting the constant weight of the crucible and ash. The weight of the ash divided by the initial weight of the drug and multiplied by a hundred was taken as the acid insoluble ash value.
3.5. ESTIMATION OF PHYTOCONSTITUENTS AND VITAMINS

3.5.1. Estimation of Total Phenol (Malick and Singh, 1980)

**Principle:** Phenols react with phosphomolybdic acid in Folin – Ciocalteau reagent in alkaline medium and produce a blue coloured complex (Molybdenum blue).

**Reagent:** Appendix IV

**Procedure:** Weighed exactly 0.5 – 1 g of the sample and grounded it with a pestle and mortar in 10 times volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 min and saved the supernatant. Re-extracted the residue with 5 times the volume of 80% ethanol, centrifuged and the supernatants were pooled. Evaporated the supernatant to dryness and dissolved the residue in a known volume of distilled water (5 ml).

The standards ranging from 0.2 – 2 ml were pipetted out into a series of test tubes. The volume in each tube was made up to 3 ml by adding distilled water. 0.5 ml of Folin – Ciocalteau reagent was added to all the tubes. After 3 min, added 2 ml of 20% sodium carbonate solution to each tube. The tubes were mixed thoroughly and the tubes were kept in a boiling water bath for exactly one minute, the tubes were then cooled and the absorbance was measured at 650 nm against a reagent blank. A standard curve was prepared using different concentration of catechol. From the standard graph the concentration of phenol in test sample were found out and expressed as mg phenol/100 g of plant material.

3.5.2. Estimation of Tannin by Folin – Denis Method (Schanderi, 1995)

**Principle:** Tannin – like compounds reduce phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution, the intensity of which is proportional to the amount of tannins. The intensity was measured in a spectrophotometer at 700 nm.

**Reagent:** Appendix V

**Procedure:** Extraction of tannin- weighed 0.5 g of plant sample and transferred to 250 ml conical flask and added 75 ml of distilled water. Heated the flask gently and boiled for
30 min. Centrifuged at 2000 rpm for 20 min and collected the supernatant in 100 ml volumetric flask and made up to 250 ml.

**Estimation:** 1 ml of the sample extract was transferred to 100 ml flask containing 75 ml of distilled water. A blank was prepared with distilled water. 5 ml of Folin – Denis reagent was added to all the tubes followed by 10 ml of sodium carbonate solution. All the tubes were diluted to 100 ml with distilled water and shook well.

The absorbance was read at 700 nm after 20 min. A standard graph was prepared using 0 – 100 µg tannic acid. The tannin content was expressed as tannic acid equivalents.

3.5.3. Estimation of Saponin (Hudson and Ei-Difrawi, 1979)

**Reagents:** Appendix VI

**Procedure:** About 10 g of sample was taken in a 100 ml volumetric flask containing 20% aqueous ethanol in water. The mixture was agitated with a magnetic stirrer for 12 hrs at 55°C. The solution was filtered using whatman No.1 filter paper and the residue was re-extracted with 200 ml of 20% aqueous ethanol. The extracts were combined and reduced to 40 ml under vacuum using a rotary evaporator. The extract and 20 ml diethyl ether were transferred into 250 ml separating funnel and was shaken vigorously. The aqueous layer was discarded. The purification process was continued until a colourless aqueous extract was obtained. The pH of the aqueous solution was adjusted to about 4.5 by adding 4 g of sodium chloride and the solution was shaken with butanol. This extract was then washed twice with 10 ml of 5% sodium chloride and evaporated to dryness in a fume cupboard to give saponin. The residue was then weighed and expressed in percentage.

Reagent: Appendix VII

5 g of plant sample was weighed into 250 ml beaker and 200 ml of 20% acetic acid in ethanol was added and covered to stand for 4 hours. This was filtered and the extract was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitation was collected by filtration and weighed.

3.5.5. Estimation of Flavonoid (Boham and Kocipai, 1994).

Reagent: Appendix VIII

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatmann filter paper No.1. The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed.

3.5.6. Estimation of Protein by Lowry’s Method (Lowry et al., 1951)

Principle: The blue colour developed by the reduction of the phosphomolybdic – phosphotungstic compounds in the Folin – Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein and the colour developed by the reaction of the protein with alkaline cupric tartarate present in the biuret reagent was measured by the Lowry’s method.

Reagent: Appendix IX

Procedure: Extraction was usually carried out with buffers used for the enzyme assay. Weighed 500 mg of the sample and grounded well with a pestle and mortar in 5 – 16 ml of the buffer, centrifuged and used the supernatant for protein estimation.

Estimation of Protein: Pipetted out 0.2 – 1 ml of working standard and 0.1, 0.2 ml of the sample extracts into a series of test tubes. The volume of the test tubes was made up to 1 ml. A tube with 1 ml of distilled water alone served as blank. To this added 5 ml of
reagent C to all the tubes including blank. Mixed well and allowed to stand for 10 minutes. Then added 0.5 ml of reagent D to the tubes and mixed well. The tubes were incubated at room temperature for 30 minutes. Blue colour developed was read at 660 nm. The concentration of protein was expressed as mg/24 hrs samples.

3.5.7. Estimation of Total Carbohydrate by Anthrone Method (Hedge and Hofreiter, 1962)

**Principle:** Carbohydrates were first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose was dehydrated to ethyl hydroxyl methyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

**Reagents:** Appendix X

**Procedure:** Weighed 100 mg of the sample into a boiling tube hydrolysed by keeping it in boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cooled at room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the volume to 100 ml, centrifuged and used aliquots for analysis.

Prepared the standards and made up the volume to 1 ml with distilled water. 4 ml of anthrone reagent was added. Heated for 8 minutes in a boiling water bath, cooled rapidly and read the absorbance at 630 nm.

3.5.8. Estimation of Tocopherol (Vitamin E) (Rosenberg, 1992)

**Principle:** Tocopherol can be estimated using Emmerie – Engel reaction which is based on the reduction of ferric ions to ferrous ions by tocopherols, which then forms a red colour with 2, 2’ – dipyridyl. Tocopherols and carotenoids were first extracted with xylene and the extinction read at 460 nm to measure carotenoids. A correction for the carotenoids was made after adding ferric chloride and read at 520 nm colorimetrically.
Reagents: Appendix XI

Extraction: 2.5 g of the sample was weighed and homogenized in a conical flask. 50 ml of 0.1 N sulphuric acids was added slowly without shaking. Stoppered and allowed to stand overnight. On the next day the contents were shaken vigorously and filtered through Whatmann No. 1 filter paper, discarding the initial 10 – 15 ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure: Into 3 stoppered centrifuge tubes pipette out 1.5 ml of extract and 1.5 ml of water respectively. To the test and blank 1.5 ml of ethanol was added and to the standard 1.5 ml of water was added and centrifuged. Transferred 1ml of xylene layer to another stoppered tube, taking care not to include any ethanol or protein. 1.0 ml of 2, 2’ dipyridyl reagent was added to each tube, stoppered and mixed. 1.5 ml of the mixture was pipetted out to the colorimeter cuvettes and read the extinction of the test and standard against the blank at 460 nm. To the blank added 0.3 ml of ferric chloride solution, mixed well and exactly after 15 minutes the test and standard were read against the blank at 520 nm.

3.5.9. Estimation of Ascorbic Acid (Roe and Keuther, 1953)

Principle: Ascorbate was converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbate then reacts with 2, 4, - Dinitro phenyl hydrazine to form osazones, which dissolved in sulphuric acid to give an orange coloured solution whose absorbance measured spectrophotometrically at 540 nm.

Reagents: Appendix XII

Extraction: 1 g of sample was ground and homogenized using 10 ml of 4% TCA centrifuged at 2000 rpm for 10 min. The supernatant obtained was treated with a pinch of activated charcoal residue. Shaken well and kept for 10 minutes. Once again centrifugation was carried out to remove the charcoal residue. The volume of the clear supernatant obtained was noted.
**Procedure:** 0.5 ml and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made up to 2 ml with 4% TCA. 0.2 – 1.0 ml of working standard solution containing 20-100 µg ascorbate respectively were pipetted out into clean dry test tubes and the volumes were made up to 2 ml with 4% TCA. 0.5 ml of DNPH reagent was added to all the tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hours. The osazones formed were dissolved in 2.5 ml of 85% sulphuric acid in cold, drop by drop, with no appreciable rise in temperature. To the blank, DNPH reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30 min at room temperature, the absorbance was read spectrophotometrically at 540 nm.

**3.5.10. Estimation of Riboflavin (B_{1}) (Poornima and Ravishankar, 2009).**

**Reagent: Appendix XIII**

5 g of the plant sample was extracted with 100 ml of 50% ethanol solution and shaken for one hour. This was filtered into a 100 ml flask; 10 ml of the extract was pipetted into 50 ml volumetric flask. 10 ml of 5% potassium permanganate and 10 ml of 30% H_{2}O_{2} were added and allowed to stand over a hot water bath for about 30 min. 2 ml of 40% sodium sulphate was added. This was made up to 50 ml mark and the absorbance measured at 510 nm in a spectrophotometer.

**3.5.11. Estimation of Thiamin (B_{2}) (Poornima and Ravishankar, 2009)**

**Reagent: Appendix XIV**

5 g of the plant sample were homogenised with ethanolic sodium hydroxide (50 ml). It was filtered into a 100 ml flask. 10 ml of filtrate was pipetted and the colour developed by addition of 10 ml potassium dichromate and read at 360 nm. A blank sample was prepared and the colour also developed was read at the same wavelength.
PHASE II

3.6. HPTLC ANALYSIS OF *Barleria cristata* L. CRUDE LEAF EXTRACT FOR SAPONIN PROFILE (Wagner and Bladt, 1996)

Reagents: Appendix XV

3.6.1. Extraction of sample

The dried leaf sample 5 g was extracted with 100 ml methanol in soxlet apparatus for 6 hours and filtered the extract through Whatman No.1 filter paper. The crude extracts were condensed by using vacuum evaporator. The contents were dissolved in 20 ml methanol. This solution was used as test solution for High performance thin layer chromatography (HPTLC) analysis.

3.6.2. Sample loading

The test solution of 8 µl was applied as 10 mm band on 5 × 10 precoated silica gel (60 F$_{254}$). Thin layer chromatography (TLC) plate of uniform thickness of 0.2 mm using Linomat 5 systems.

3.6.3. Spot Development

The sample loaded TLC plate was kept in TLC twin trough developing chamber with respective mobile phase (for saponins separation) for 15 minutes (for chamber saturation). The plates were developed up to 8 cm with mobile phase. The plates were removed and allowed to dry in air.

3.6.4. Photo – Documentation

The developed plates were dried by hot air to evaporate solvents from the plate. The plates were kept in photo – documentation chamber and were viewed under white light, UV 254 nm and UV 366nm wave length light in Reprostar – 3.
3.6.5. Derivatization

The plates were sprayed with respective spraying reagent for saponins and dried at 120°C for 5 min in a hot air oven. The compounds were identified and immediately the plates were photodocumented using Reprostar – 3.

3.6.6. Scanning

The plate was scanned at 500 nm for saponins using TLC scanner – 3 after derivatization. The peak areas were observed from the peak table.

3.7. HPLC ANALYSIS OF Barleria cristata L. LEAF EXTRACT (crude and 50% hydroethanolic extract) FOR SAPONIN IDENTIFICATION USING Bacopa (BRAHMI) TABLETS AS STANDARD

Principle:  Bacopa monnieri  saponins from Bacopa tablets were identified in the leaf extract of Barleria cristata L. plant by HPLC at 205 nm.

Reagents: Appendix XVI

3.7.1. Preparation of Commercial Brahmi tablet: Weighed accurately 1.6718 gm of the powdered tablet in a 100 ml conical flask and dissolved with 30 ml of sulphate buffer: acetonitrile in the ratio of 1:1. Allowed to stand for 2 hrs with frequent shaking. Filtered the solution and transferred the content into a 50 ml standard flask and diluted up to the mark with the sulphate buffer: acetonitrile in the ratio of 1:1 (668.72 µg/20 µl).

3.7.2. Extraction of saponin from Barleria cristata L.

Weighed accurately 2.2016 gm of the powdered crude leaf material in a 100 ml round bottom flask, added 30 ml of 70% methanol (v/v) and refluxed for 1 hr. Filtered and the residue was re -extracted with 20 ml of 70% methanol for 30 minutes. Combined all the extracts, condensed the extracts using flash evaporator under vacuum. Dissolved 104.2 mg of the extract using sulphate buffer: acetonitrile, in the ratio of 1:1 and transferred the contents to a 25 ml standard flask, diluted up to the mark with sulphate buffer: acetonitrile in the ratio of 1:1 (83.36 µg/µl).
3.7.3. Preparation of *Bacopa monnieri* saponin from 50% hydroethanolic extract of *Barleria cristata* L.

Weighed accurately 1.6166 gm of the 50% hydroethanolic extract of *Barleria cristata* L. Into a 50 ml standard flask, dissolved using 20 ml of sulphate buffer: acetonitrile in the ratio of 1:1 and diluted up to the mark with the sulphate buffer: acetonitrile in the ratio of 1:1 (646.64 µg / 20µl).

**Procedure:** The HPLC separation was performed using a shimadzu HPLC system. A phenomenex C18 (250 × 4.6 mm SS, 5 micron particle size) column was used. Ultraviolet (UV) 205 nm detector and with Spinchrom software was equipped with the HPLC. The mobile phase consisted of sodium sulphate buffer (pH 2.3): acetonitrile in the ratio (68.5: 31.5). The flow rate was 1.0 ml/min. The sample was injected with 20 µl capacity micro syringe. The elutes were monitored at 205 nm with the detector. The peaks were initially assigned by comparing retention times with the standards and confirmed with characteristic spectra obtained from the PDA. The purity of the peak was also confirmed by the detector. Calibration curves were prepared based on peak areas of plant material and 50% alcoholic extract with the standard peaks. All the data were processed using spinchrom software.

3.8. FREE RADICAL SCAVENGING ASSAY (INVITRO)


**Principle:** Antioxidants react with DPPH and convert it to diphenyl – picryl hydrazine. The degree of discoloration from purple to yellow colour was measured at 518 nm, which is a measure of the scavenging potential of antioxidant extracts.

**Reagents:** Appendix XVII

**Procedure:** To 0.5 ml of ethanolic solution of 0.3 mM DPPH, added 20 µl of 50% hydroethanolic extracts of *Barleria cristata* L. and *Rubia cordifolia* at various concentrations (100, 200, 300, 400, 500 µg). The mixture was allowed to stand at room temperature for about 30 minutes. 0.5 ml of ethanol and 20 µl of plant extracts served as
blank. DPPH in ethanol without the plant extracts served as positive control. After 30 minutes, the discoloration was measured at 518 nm in a visible spectrophotometer.

3.8.2. Superoxide Radical Scavenging Assay (Gulcin et al., 2005)
Principle: This assay was based on the inhibition of the reduction of nitroblue–tetrazolium (NBT) by the superoxide ions generated by phenazine methosulphate. The inhibitory effect was due to scavenging of the superoxide radicals by the plant constituents leading to decolorization of the reaction mixture which was measured spectrophotometrically at 530 nm.

Reagents: Appendix XVIII
Procedure: The scavenging activity of the superoxide radical was measured in terms of generation of O$_2^–$. The reaction mixture consisting of 1 ml of Nitro Blue Tetrazolium (NBT) solution (156 mM NBT in phosphate buffer, pH 7.4), 1 ml NADH solution (468 mM NADH in phosphate buffer, pH 7.4) and 1 ml of the sample solution was mixed. The reaction was started by adding 100 µl of Phenazine Methosulphate (PMS) solution (60 mM PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm against blank. The sample was compared with the standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging.

3.8.3. Nitric oxide Radical Scavenging Assay (Green et al., 1982)
Principle: Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that was estimated spectrophotometrically at 546 nm.

Reagents: Appendix XIX
Procedure: The reaction mixture containing 2 ml of 25 mM sodium nitroprusside, 0.5 ml of phosphate buffered saline (50 mM, pH 7.4) and 0.5 ml of different concentrations (100, 200, 300, 400, 500 µg/ml) of plant extracts were incubated at 25°C for 5 hrs. Control experiment without the test compound but with equivalent amount of buffer was
added in an identical manner. After 5 hrs, 0.5 ml of the incubation solution was removed from each tube and diluted with 0.5 ml of Griess reagent, the absorbance of the chromophore formed during diazotization coupling with naphtyl ethylene diamine was read at 546 nm.

3.8.4. Hydrogen Peroxide Scavenging Assay (Ruch et al., 1989)

**Principle:** The ability of the extracts to scavenge hydrogenperoxide (H$_2$O$_2$) was assessed by the method of Ruch *et al.,* (1989).

**Reagents: Appendix XX**

**Procedure:** Hydrogen peroxide solution (2 mM) was prepared in phosphate buffered saline (50 mM, pH 7.4). Ascorbic acid solution was used as standard. To 1 ml of the plant extract added H$_2$O$_2$ solution (0.6 ml) and absorbance of the hydrogen peroxide at 230 nm was read after 10 minutes against a blank solution containing extract (1ml) in PBS without H$_2$O$_2$.

Calculation of % inhibition = I % was calculated using the formula,

\[ I \% = \frac{O.D \text{ of control} - O.D \text{ of test}}{O.D \text{ of control}} \times 100 \]

3.9. *in vitro* ANTIOXIDANT POTENTIAL OF *Barleria cristata* L. AND *Rubia cordifolia* LINN.

3.9.1. Estimation of Total Carotenoids and Lycopene (Ranganna, 1976)

**Principle:** The total carotenoids in the samples were extracted in petroleum ether and estimated in a UV visible spectrophotometer at 450 nm. Lycopene has absorption maximum at 473 and 503 nm. A rapid method for the estimation of lycopene in the plant product was based on the measurement of absorption of the petroleum ether extract of the total carotenoids at 503 nm (molar co-efficient for lycopene at 503 nm = 17.2 × 10)

After measuring the total carotenoids at 450 nm, the same extract can be used for estimating the lycopene at 503 nm. At 503 nm, lycopene has maximum absorbance, while carotenes have only negligible absorbance.
**Reagents: Appendix XXI**

**Procedure:** Weighed 5-10 g of the plant samples and saponified for about 30 minutes in a shaking water bath at $37^\circ$C after extracting the sample in 12% alcoholic potassium hydroxide. Transferred the saponified extract into a separating funnel (packed with glass wool and calcium carbonate) containing 10-15 ml of petroleum ether and mixed gently. The carotenoid pigments were separated into the petroleum ether layer. The lower aqueous phase was transferred to another separating funnel. The petroleum ether extracts containing the carotenoid pigments were transferred into amber-coloured bottle. Extraction of the aqueous phase similarly with petroleum ether was repeated, until it became colourless. The aqueous phase was discarded. To the petroleum ether extract, added a small quantity of anhydrous sodium sulphate to remove turbidity. Noted the final volume of the petroleum ether extract and diluted, when needed by a known dilution factor. The absorbance of the extract at 450 and 530 nm was measured in a spectrophotometer using petroleum ether as a blank.

**3.9.2. Estimation of Reduced Glutathione (Ellman, 1959)**

**Principle:** Reduced glutathione is measured by its reaction with DTNB (5,5 – dithiobisnitro benzoic acid) (Ellman’s reaction) which produces yellow colour product, intensity of the colour developed was read at 412 nm.

**Reagents: Appendix XXII**

**Procedure:** 1 ml of 10% plant tissue homogenate was mixed with 0.4 ml of 10% TCA and centrifuged. Supernatant was assayed for GSH content by using Ellman’s reagent. 1 ml of supernatant was treated with 0.5 ml of Ellman’s reagent and 3 ml of phosphate buffer (pH 8.0). The absorbance was read at 412 nm against the reagent blank. A set of standards were also treated in the same manner.
3.9.3. Estimation of Total Antioxidant Activity

3.9.3.1. Ferric Thiocyanate (FTC) method (Kikuzaki and Nakatani, 1993)

**Principle:** The FTC method was used to determine the amount of peroxide formed at the beginning of lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate whose colour was read at 500 nm.

**Reagents:** Appendix XXIII

**Procedure:** 4 mg of plant sample and 2.52 % linolenic acid were dissolved in 4 ml and 4.1 ml of absolute ethanol. Then 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water were taken in a screw caped vial and placed in a dark oven at 40°C. To 0.1ml of this solution added 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely after 3 minutes after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of red colour was measured at 500 nm every 24 hours until one day after the absorbance of control reached its maximum. Ascorbic acid was used as positive control, while a mixture without a plant sample was used as negative control.

3.9.3.2. Thiobarbituric Acid (TBA) method (Ottolenghi, 1959)

**Reagents:** Appendix XXIV

**Procedure:** 2 ml of 20% trichloroacetic acid and 2 ml of 0.67% of 2-thio barbituric acid were added to 1 ml of sample solution. The mixture was placed in a boiling water bath and after cooling, the tubes were centrifuged at 3000 rpm for 20 minutes. Absorbance of the supernatant was measured at 552 nm. Antioxidant activity of the plant extracts were based on the absorbance on the final day of the FTC method.
3.10. Preparation of 50% hydroethanolic extracts of *Barleria cristata* L. and *Rubia cordifolia* Linn.

**Reagents: Appendix XXV**

About 5 kg of plant materials were shade dried for five days and pulverized using a mixer grinder. The coarse powder was used for the preparation of the extract.

The coarse powder was cold macerated with ethanol (i.e. 2 litres of ethanol and 2 liters of water) for 3 days, with occasional stirring. After 3 days the suspension was filtered through a fine muslin cloth and the residue was removed. Then the water portion of the samples was evaporated at a low temperature (approximately at 40°C) under reduced pressure in a rotary evaporator. Dark brown coloured crystals of 0.7 and 0.9 g was obtained. The crystals were stored in an air-tight dessicator and whenever needed, the residues were dissolved in distilled water, filtered (whatman No.1 filter paper) and used for the studies.

3.11. Acute Toxicity Studies (Miller and Tainter, 1944)

The determination of ED$_{50}$ (the dose effective in producing expected response in 50% animal group) values help in ascertaining the potency of a drug in terms of reference standard. The calculation of ED$_{50}$ value was done when a drug show graded response in relation to dose. But when the response was quantal or all or none, the ED$_{50}$ value became LD$_{50}$ (the dose lethal to 50% of the animal groups). The ED$_{50}$ and LD$_{50}$ values were important for knowing the safety of a drug. The ratio between ED$_{50}$ and LD$_{50}$ represented the therapeutic index which was an index of safety of the drug. Greater the therapeutic index, safer was the drug.

LD$_{50}$ was calculated by finding the least tolerated (smallest) dose (100% mortality) and most tolerated (highest) dose (0% mortality) by hit and trial method. Once the two does were determined at least 5 doses were selected in between the least tolerated and most tolerated dose and the mortality due to these doses was observed. LD$_{50}$ value of
a new drug can be determined by either oral or any one of the parenteral routes 
(intraperitoneal, intravenous and intramuscular) of administration.

Wistar albino rats were fasted overnight, weighed and divided into 6 groups, each 
group consisting of five animals. Test samples were administered separately in various 
doses by oral route (2000, 4000, 6000, 8000, 10000 mg/kg body wt). After administration 
of the compounds, the animals were observed continuously for the first two hours for 
death due to acute toxicity. The results of LD$_{50}$ study were analyzed using Miller and 
Tainter method. The LD50 was calculated graphically and theoretically. First, the 
percentage of death was computed and then corrected percent for 0% and 100% was 
calculated using the formula

\[
\begin{align*}
0\% \text{ death} &= (0.25/n) \times 100 \\
100\% \text{ death} &= (n - 0.25)/n \times 100
\end{align*}
\]

Where

\[n \text{ – Number of animals in each group.}\]
3.12. ANTILIPIDEMIC STUDY

3.12.1 Experimental animals

Male albino rats of Wistar strain weighing 120 – 150 g were procured from the animal house, PSG Institute of Medical Science and Research (No: 158/1999/CPCSEA), Coimbatore, India. The rats were grouped and housed in polyacrylic cages and maintained under standard conditions (25 ± 2°C) with 12 ± 1 hours dark/light cycle. The animals were fed with rat pellet feed supplied by Hindustan Lever Ltd., Bangalore, India and water ad libitum. All the experimental procedures were conducted after the approval of ethical committee and were in strict accordance with institutional animal ethical committee guidelines for the care and use of laboratory animals.

3.12.2. Induction of diabetes

Alloxan monohydrate was used to induce diabetes mellitus in normoglycemic rats. Animals were allowed to fast for 18 hours and were injected intraperitoneally with freshly prepared alloxan monohydrate in sterile normal saline in a dose of 120 mg/kg body weight. After 72 hours of injection, 0.5 to 1 ml of blood was collected from the rats using microcapillary tube by retro-orbital route. The diabetic rats with fasting blood glucose levels greater than 350 mg/dl approximately were selected for the study.

Experimental Setup

The overnight fasted rats were divided into 6 groups of 6 animals each.

Group I – Served as normal healthy controls and received standard rat pellet for 28 days.

Group II – Diabetic rats served as diabetic control

Group III – Diabetic rats received glibenclamide (600 µg/kg body weight) drug for 28 days served as drug control.

Group IV – High Lipid Diet (HLD) Control rats fed with high lipid diet for 28 days.

Group V – HLD rats received 50% hydroethanolic leaf extract of *Barleria cristata* L. (200 mg / kg b.w) for 28 days.
Group VI - HLD rats received 50% hydroethanolic leaf extract of *Barleria cristata* L. (400 mg / kg b.w) for 28 days.

Group VII - Diabetic rats received 50% hydroethanolic leaf extract of *Barleria cristata* L. (500 mg / kg b.w) for 28 days.

3.12.3. Induction of Hyperlipidemia

High lipid diet was prepared by mixing 50 g fat as Dalda, 32 g whole Wheat flour, 16 g of whole Milk powder, 1g NaCl, 1g Cholesterol, 0.2 mg Thiamine, 0.25 mg Riboflavin and 3 mg Niacin. Rats of group IV, V and VI were fed with HLD for 30 days and their serum cholesterol levels were checked after 30 days. When the cholesterol level reached 350 – 400 mg/dl, group V and VI rats were subjected to further plant extract treatment.

Reagents: Appendix XXVI

3.12.4. Blood and Tissue sample collection

At the end of treatment period (28 days), blood sample of about 1 – 1.5 ml were collected after mild chloroform anaesthesia and by the cardiac puncture. Serum was collected from blood by centrifuging at 3000 rpm for 20 minutes at room temperature (for enzyme assays blood was centrifuged at 4°C).

The liver and kidneys were excised quickly and blotted with ice cold saline, dried and stored at 4°C till all the analysis was done.

3.12.5 Preparation of Tissue homogenate:

One gram of liver and kidney were taken and homogenized with 10 ml of 0.1 M cold Tris HCl buffer (pH 7.4) in a homogenizer fitted with Teflon plunger at 600 rpm for 3 minutes. The homogenate was used for various biochemical assays.
3.12.6 Determination of Serum Lipid Profile.

3.12.6.1 Estimation of Cholesterol (Richmond, 1973)

**Principle:** Cholesterol was determined after the enzymatic hydrolysis and oxidation process. Cholesterol esters were hydrolysed by the Cholesterol esterase to give free cholesterol and fatty acid molecules. This free cholesterol gets oxidized in presence of Cholesterol Oxidase to liberate Cholest-4-ene-3-one and Peroxide, the indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The intensity of this coloured complex was measured at 505 nm (500 – 540 nm) and this was directly proportional to the cholesterol concentration present in the sample.

**Reagents: Appendix XXVII**

**Procedure:** Reagents were pipetted out into three test tubes labeled Blank (B) Standard (S) and T (Test) as given below.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Blank (µl)</th>
<th>Standard (µl)</th>
<th>Test (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Enzyme reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Serum/plasma</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixed well and incubated the tubes for 5 mins at 37°C. Absorbance of standard (S) and test (T) were read against the blank (B) at 505 nm.

3.12.6.2. Estimation of HDL – Cholesterol (Castelli et al., 1977)

**Principle:** HDL – Cholesterol Reagent reacts directly with LDL and VLDL at pH 10 to form insoluble complexes. This action occurs at room temperatures. The precipitate can be removed by centrifugation and the supernatant was analysed for HDL Cholesterol.
**Reagents: Appendix XXVIII**

**Procedure:** Reagents were pipetted out into 3 test tubes labeled Blank (B), Standard (S) and Unknown (U) as given below

<table>
<thead>
<tr>
<th>Content</th>
<th>Blank (µl)</th>
<th>Standard (µl)</th>
<th>Unknown (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Unknown Sample</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

The tubes were mixed thoroughly and incubated at 37°C for 5 mins. The absorbance was measured at 505 nm.

**Calculations:**

Concentration of Triglyceride (mg/dl)  = \( \frac{\text{Absorbance of } T_c \times 200}{\text{Absorbance of } S} \)

Concentration of HDL Cholesterol (mg/dl)  = \( \frac{\text{Absorbance of } T_{hl} \times 50}{\text{Absorbance of } S} \)

**3.12.6.3. Estimation of Triglycerides (Philip and Mayne, 1994)**

**Principle:** Triglyceride was determined after the enzymatic hydrolysis with lipase. Serum triglycerides were hydrolysed to glycerol and free fatty acids by lipases. In the presence of ATP and glycerol kinase, glycerol was converted to glycerol 3 phosphate which was then oxidized by GPO to yield hydrogen peroxide. Peroxidase catalyses the conversions of hydrogen peroxide, 4 amino antipyrine and ESPAS to a purplish brown coloured quinonime complex which was measurable at 546 nm.

\[
\begin{align*}
\text{Lipases} & \quad \text{Triglycerides} + H_2O \quad \rightarrow \quad \text{Glycerol} + \text{Fatty acids} \\
\text{Glycerol Kinase} & \quad \text{Glycerol} + \text{ATP} \quad \rightarrow \quad \text{Glycerol 3 phosphate (G-3-P)} + \text{ADP} \\
\text{GPO} & \quad \text{G-3-P} + O_2 \quad \rightarrow \quad H_2O_2 + \text{Dihydroxy acetone phosphate} \\
\text{POD} & \quad H_2O_2 + 4\text{-aminoantipyrine} + \text{ESPAS} \quad \rightarrow \quad \text{Brown Quinoneimine} + H_2O + HCl
\end{align*}
\]
**Reagent: Appendix XXIX**

**Procedure:** Reagents were pipetted into 3 test tubes labeled Blank (B), Standard (S) and Test (T) as given below.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Blank (µl)</th>
<th>Standard (µl)</th>
<th>Test (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triglyceride standard</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Serum/plasma</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixed well and incubated the tubes for 10 mins at 37°C. Absorbance of standard and test were read against the blank at 546 nm (540-560 nm). The final colour, brownish purple was stable for 30 mins at room temperature.

**Calculations:**

Concentration of Triglycerides (mg/dl) = \( \frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times 200 \)

The LDL, VLDL, values were calculated by Friedewald's formula as given below (Friedewald et al., 1972).

\[
\begin{align*}
\text{VLDL} & = \frac{\text{TG}}{5}, \\
\text{LDL} & = \text{TC} - (\text{HDL} + \text{VLDL}).
\end{align*}
\]

**ANTIDIABETIC STUDIES**

3.13. Antidiabetic Activity of *Barleria cristata* L. stem extract

3.13.1. Treatment groups

The experimental rats were divided into 5 groups of 6 animals in each group, after two weeks of acclimatization period.

- **Group I** - Normal Control
- **Group II** - Diabetic control
- **Group III** - Drug Control (glibenclamide 600 µg/kg body weight)
Group IV - Diabetic rats received 50% hydroethanolic extract of *Barleria cristata* L. stem (200 mg/kg body weight)

Group V - Diabetic rats received 50% hydroethanolic extract of *Barleria cristata* L. stem (400 mg/kg body weight)

Reagents: Appendix XXX

### 3.13.2 Blood and Tissue sample collection

At the end of treatment period (28 days), blood sample of about 1 – 1.5 ml were collected after mild chloroform anesthesia and by the cardiac puncture. Serum was collected from blood by centrifuging at 3000 rpm for 20 minutes at room temperature (for enzyme assays blood was centrifuged at 4\(^\circ\)C).

The liver and kidneys were excised quickly and washed with ice cold saline, dried by blotting and stored at 4\(^\circ\)C till all the analysis were done. A portion of liver, kidney and pancreas were cut from each group and fixed in 10% formalin solution for histopathological studies.

### 3.13.3 Preparation of Tissue homogenate:

One gram of liver and kidney were taken and homogenized with 10 ml of 0.1 M cold Tris HCl buffer (pH 7.4) in a homogenizer fitted with Teflon plunger at 600 rpm for 3 minutes. The homogenate was used for various biochemical assays.

### 3.13.4 Biochemical analysis

#### 3.13.4.1 Estimation of Serum Glucose (Trinder, 1969)

The serum glucose was determined by GOD/POD kit method.

**Principle:** Glucose was oxidised by glucose oxidase (GOD) to liberate D-gluconic acid and hydrogen peroxide. The colorimetric indicator, quinoneimine was generated after the addition of 4 – amino antipyrine and phenol, by reaction with hydrogen peroxide under the catalytic action of peroxidase. Intensity of colour generated was directly proportional to the glucose concentration.

\[
\text{D - glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{GOD}} \text{D- gluconic acid} + \text{H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + 4 \text{ amino antipyrene} + \text{phenol} \xrightarrow{\text{POD}} \text{Red Quinoneimine dye} + 4 \text{H}_2\text{O}
\]
Reagents: Appendix XXXI

Procedure: Three clean dry test tubes were labeled as Blank (B), Standard (S) and Test (T). The reagents were pipetted out as indicated below.

<table>
<thead>
<tr>
<th>Content</th>
<th>B (µl)</th>
<th>S (µl)</th>
<th>T (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose standard</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>Serum/plasma</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Mixed well and incubated at 37°C for 15 minutes. The color formed was measured in a spectrophotometer at 404 nm.

Calculations:

Concentration of Glucose in mg % = \( \frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times 100 \)

3.13.4.2. Estimation of Liver Glycogen (Vander Vries, 1954)

Principle: Glycogen was treated with anthrone reagent to give a green colored complex which was read at 650 nm.

Reagents: Appendix XXXII

Extraction of glycogen: 200 mg of liver samples were weighed and ground with 20 ml of 5% TCA in a homogenizer. The precipitate of protein was filtered and clear filtrate was used for estimation.

Procedure: 2 ml of liver extract and 2 ml of 10 N KOH were pipetted into a test tube and kept in boiling water both for 1 hr. After cooling the tubes, 1 ml of glacial acetic acid was added to neutralize the excess alkali. To 2 ml of this solution, added 4 ml of anthrone reagent in cold conditions. The tubes were mixed well and kept in a boiling water bath for 10 min. The color developed was read at 650 nm. 2 ml of 5% TCA and 4 ml of anthrone reagent together served as blank.
3.13.4.3. Estimation of Insulin (UBI - MAGIWEL – Enzyme Immuno Assay method (Clark and Hales, 1991)

**Principle:** UBI – MAGIWEL insulin assay was a solid phase Enzyme linked Immuno sorbant Assay (ELISA) method. The wells were coated with monoclonal antibodies with higher affinity for insulin when the samples and controls were incubated in the wells with enzyme conjugated antibody linked to horse radish peroxidase, a sandwich complex was formed and was bound to the well. The unbound conjugate was washed off with wash buffer. The amount of bounded peroxidase was directly proportional to the concentration of the insulin present in the sample. Upon addition of the hydrogen peroxide and tetramethylbenzidine the color was developed. The intensity of color developed was proportional to the concentration of insulin in the sample which was read at 450 nm.

**Reagents: Appendix XXXIII**

**Assay Procedure:** Into the coated well added 25 µl of serum sample, control and reference (human insulin). Added 100 µl of enzyme conjugate (anti-insulin antibodies conjugated to horse radish peroxidase) into each well and mixed for 5 seconds. The tubes were incubated for 30 mins at 25°C. Removed the incubation mixture and rinsed the wells for five minutes using washing buffer. 100 µl solution A (buffer solution containing hydrogen peroxide) and solution B (tetramethyl benzidine) was added into the each well and incubated for 15 min at room temperature. The reaction was stopped by adding 50 µl of 2N hydrochloric acid to each of the wells and the optical density was read at 450 nm with a micro-well reader.

3.13.4.4. Estimation of Glycosylated Haemoglobin (Saibene et al., 1979; Jim and Phillip, 1983)

The term glycosylated haemoglobin (gHb) usually refers to the series of minor glycated fractions of Hb. The erythrocyte was freely permeable to glucose. Glycosylated Hb was formed at a rate dependent on the average glucose concentration and increases in animals with diabetes mellitus.
**Reagents: Appendix XXXIV**

**Procedure:** Red blood cells were separated from 4 ml of blood after the addition of EDTA and by centrifuging at 1400 x g for 15 min. After triple washing with saline, 1 ml of RBC was haemolysed using water and 0.4 ml of toluene. Following mixing, toluene and the debris were removed by centrifugation (1400 gm for 20 min) and filtrated. Hexose bound to Hb was hydrolysed by heating at 100°C for four hours in the presence of oxalic acid (1 ml of oxalic acid in 2 M of Hydrochloric acid 1: 2). After cooling, 4 ml 40% TCA was added to the mixture for precipitation of protein and centrifuged at 2000g for 10 min. Acid was added to 6 ml of the supernatant followed by 2 ml of 0.05M 2-thiobarbituric acid and after 40 min of incubation at 40°C, the color developed was read at 443 nm against a reagent blank.

**3.13.4.5. Estimation of Hexokinase (Brandstrup et al., 1957)**

**Principle:** The hexokinase enzyme was assayed by determining the rate of disappearance of glucose at 37°C.

**Reagents: Appendix XXXV**

**Procedure:** The incubation mixture containing 2.5 ml Tris-HCl buffer, 1.0 ml of substrate, 0.5 ml of ATP, 0.1 ml of megnesium chloride, 0.1 ml of sodium fluoride, 0.5 ml of potassium dihydrogen phosphate and 0.5 ml of potassium chloride were pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of 2 ml of enzyme extract. 1 ml aliquot of the reaction mixture was removed immediately (0 time) and added to the tubes containing 1 ml of 10% TCA. After 30 min of incubation, the reaction was stopped by the addition of 1.0 ml of 10% TCA. The samples were used for the estimation of glucose by ortho-toludine method. The enzyme activity was expressed as µmoles of glucose utilized for the formation of glucose 6-phosphate/min/mg protein.

**Estimation of Glucose by Ortho-Toludine (OT) Method (Dubowski, 1962)**

**Principle:** O-toludine 6% (w/v) in glacial acetic acid was used to determine glucose in biological materials after deproteinization with 3% (w/v) trichloroacetic acid. A stable
green color developed after heating at 100°C for 10 min and the absorbance was determined at 630 nm.

**Reagents: Appendix XXXVI**

**Procedure:** The sample was deproteinized by adding 1.8 ml of 3% trichloroacetic acid. Allowed to stand (5-10 min) and filtered through a whatmann No.2 filter paper. 1ml of distilled water (blank) and 1ml of sample were taken in two test tubes. To this added 3 ml of O-toludine reagent to each tube and mixed well by repeated inversion. The tubes were immersed in a 100°C fluid bath for 10 min and then removed and cooled. The absorbance of the test sample was measured in a spectrophotometer at 630 nm.


**Principle:** Urea directly reacts with diacetyl monoxime in a strong acidic condition to produce a yellow condensation product. The reaction was intensified by the presence of ferric ions and thiosemicarbazide. The red colour formed was measured spectrometrically at 540 nm.

**Reagents: Appendix XXXVII**

**Procedure:** 0.2 – 1.0 ml of working standard solution was taken in series of test tubes labeled S1 – S5 and 0.2 ml of sample alone in the tube labeled ‘Test’. All the tubes were made up to 2 ml using distilled water and mixed well. The colour reagent was prepared fresh at the time of analysis by mixing distilled water, mixed acid reagent and mixed color reagent in 1:1:1 ratio. Then added 3 ml of colour reagents to all the tubes, the tubes were mixed well and kept in a boiling water bath for 15 min. After the incubation period the tubes were removed from the water bath and cooled for 5 min. The transmittance was measured in a spectrophotometer at 540 nm against reagent blank. A blank was also treated in the same manner and instead of sample, distilled water was taken.
3.13.4.7. Estimation of Creatinine by Jaffe’s method (Slot and Scand, 1965)

**Principle:** Creatinine present in the serum reacts with alkaline picrate resulting in the formation of red colour, the intensity of which was measured at 505 nm. The interference of protein was eliminated by using beryl sulphate. A second absorbance reading after acidifying with 30% acetic acid corrects for non-specific chromogens in the sample.

**Reagents:** Appendix XXXVIII

**Procedure:** Pipetted 0.2 – 1.0 ml of working standard in a series of test tubes (S1 – S5) and 0.2 ml of serum in the tube labeled as ‘Test’. The final volume was made up to 2 ml in all the tubes using distilled water. 3.0 ml of working reagent was added to all the tubes and mixed well. The tubes were left 30 min at room temperature. After incubation the transmittance was measured at 505 nm (A1). Added 0.2 ml of 30% acetic acid to the ‘Test’, tubes, mixed well and incubated at room temperature for 5 min. The transmittance was measured at 505 nm (A2). A2-A1 gives the change in absorbance, which was the measure of creatinine present in the sample. The results were expressed as mg/dl in serum.

3.13.4.8. Estimation of Aspartate Transaminase (Reitman and Frankel, 1957)

**Principle:** The enzyme catalyses the reversible inter conversions between glutamate and aspartate and their 2-oxo analogues.

\[
\text{L-Aspartate + } \alpha\text{- Oxoglutarate} \rightleftharpoons \text{Oxaloacetate + L- Glutamate}
\]

The oxaloacetate was measured by the reaction with 2,4, Dinitro phenyl hydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

**Reagents:** Appendix XXXVIX

**Extraction of enzyme:** 1 g of tissue was ground with 10 ml of 0.1M sodium phosphate buffer (pH 7.5) in a homogenizer for 20 min. The slurry was filtered through 8 layer of cheese cloth and then centrifuged at 25,000 g for 15 min. The supernatant was used for the assay.
**Procedure:** 0.2 ml of sample and 1 ml of buffered substrate (aspartate and α-ketoglutarate) were incubated for 1 hr at 37°C. To the control tubes, added enzyme after arresting the reaction with 1ml of dinitrophenylhydrazine and kept the tubes at room temperature for 20 min. After incubation 1ml of 0.4 N sodium hydroxide was added to the tubes. A set of standard pyruvate was treated in a similar manner. Read the colour developed at 540 nm. A blank was taken in the same manner as above; instead of sample distilled water was taken. The enzyme activity was expressed as units/l in serum, units /mg protein in tissues.

**3.13.4.9. Estimation of Alanine Transaminase (Reitman and Frankel, 1957)**

**Principle:** The enzyme catalyses the following reaction.

\[
\text{L-Alanine + \alpha – Oxoglutarate} \quad \text{\rightarrow} \quad \text{Pyruvate + L-glutamate}
\]

The pyruvate was measured by the reaction with 2, 4 dinitrophenyl hydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed was read at 520 nm.

**Reagents: Appendix XL**

**Extraction of enzyme:** 1 g of tissue was ground with 10 ml of 0.1M phosphate buffer (pH 7.5) in a homogenizer for 2 min. The slurry was passed through 8 layers of cheese cloth and then centrifuged at 25,000 g for 15 min. The supernatant was used for the assay.

**Procedure:** 0.2 ml of sample and 1ml of the buffered substrate (alanine and α-ketoglutarate) was incubated for 30 min at 37°C. To the control tubes, added enzyme after arresting the reaction with 1 ml of dinitrophenylhydrazine and kept the tubes at room temperature for 20 min. 1 ml of 0.4 N sodium hydroxide was added to all the tubes. A set of standard pyruvate was treated in a similar manner. Read the colour developed at 520 nm.

The enzyme activity was measured as units/l in serum, units/mg in tissues.
3.13.4.10. Estimation of Alkaline Phosphatase (King and Armstrong, 1934)

**Principle:** The method is that of King and Armstrong in which disodium phenylphosphate was hydrolyzed with the liberation of phenol and inorganic phosphate. The liberated phenol was measured at 700 nm with Follin – Ciocalteau reagent.

**Reagents:** Appendix XLI

**Extraction of enzyme:** 0.5 g of tissue was homogenized with buffer in a pre-chilled pestle and mortar, filtered through cheese cloth and centrifuged at 10,000 g for 10 min. The supernatent was used for the assay.

**Procedure:** Pipetted 4 ml of buffer substrate into the test tube and incubated at 37°C for 5 min, added 0.2 ml of serum (or) tissue homogenate and incubated for 15 min. To the ‘control’ tube alone, the homogenate was added after arresting the reaction with 0.9 ml of Follin’s phenol reagent. The contents were then centrifuged for 15 min. To 2 ml of the supernatant, 1ml of sodium carbonate was added, the tubes were again incubated for 15 min at 37°C and colour developed was read at 700 nm. Taken 2.0 ml of working standard solution and for blank taken 1.6 ml of water and 0.4 ml of phenol reagent. Then added 1ml of sodium carbonate buffer and were analysed in same manner. The enzyme activity was expressed as units/l in serum, units/mg protein in tissue.

3.13.4.11. Estimation of Acid Phosphatase (King, 1965)

**Principle:** Acid phosphatase was estimated according to King method in which disodium phenyl phosphate was hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol was measured at 700 nm with Follin Ciocalteau reagent.

**Reagents:** Appendix XLII

**Extraction:** 0.5 g of tissue was homogenized with buffer in a ice cold pestle and mortar. Filtered and centrifuged the filtrate at 10,000 g for 10 min. The supernatent was used for assay.
**Procedure:** Pipetted 2 ml of buffered substrate into a test tube and incubate at 37°C for 2 min, added 0.1 ml of the sample and incubated further for 60 min. Removed and immediately added 0.9 ml of diluted phenol reagent. At the same time set up a control with 2 ml of buffered substrate and 0.1 ml of sample to which 0.9 ml phenol reagent was added immediately. Mixed well and centrifuged. To 2 ml of the supernatant added 1 ml of sodium carbonate. Taken 2 ml of working standard solution and for blank taken 1.6 ml of water and 0.4 ml of phenol reagent. Then added 1.0 ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min and the colour developed was read at 700 nm. The enzyme activity was expressed as unit/l in serum and units/mg protein in tissue.

**3.13.4.12. Estimation of Lactate Dehydrogenase (King, 1965)**

**Principle:** The lactate was acted upon by lactate dehydrogenase to form pyruvate in the presence of NAD. The pyruvate forms phenyl hydraxone with 2, 4 DNPH. The colour developed was read in a spectrophotometer at 440 nm.

**Reagents: Appendix XLIII**

**Procedure:** In the test tube labeled ‘Test’ taken 0.5 ml of buffered substrate and 100 µl of sample. 100 µl of water was added to the tube marked as ‘Blank’. Then to the ‘Test’ tube added 0.1 ml of NAD. Mixed and incubated the tubes at 37°C for 15 min and exactly after 15 min, 0.5 ml of dinitrophenylhydrazine was added to the ‘Test’ tube. To the control tube alone, the sample was added after the addition of DNPH. The tubes were kept at room temperature for 15 min. Then added 5 ml of 0.4 N sodium hydroxide and the colour developed was read immediately at 440 nm. Pyruvate was used as standard with the concentration range of 0.1-1.0 µM. The enzyme activity was expressed as units/l in serum, units/mg protein in tissue.

**3.13.4.12. Estimation Superoxide Dismutase (SOD) (Kakkar et al., 1984)**

**Principle:** Superoxide Dismutase proportionately inhibits the formation of NADH phenazine Methosulphate NitroBlue Tetrazolium (PMSNBT) formazon. The residual chromogen can be extracted into an organic solvent like butanol and estimated at 560 nm, as a measure of SOD activity.
Reagents: Appendix XLIV

Procedure: Enzyme preparation – 0.5 ml of the sample (tissue homogenate) was diluted to 1 ml with water. To this 2.5 ml ethanol and 1.5 ml of chloroform (reagent in chilled condition) were added. This mixture was shaken for 1 minute at 4°C and then centrifuged. The enzyme activity in the supernatent was determined.

Assay: The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of Phenazine Metho Sulphate (PMS), 0.3 ml of Nitro Blue Tetrazolium (NBT), 0.2 ml of enzyme preparation and 1.2 ml of water in a total volume of 3.0 ml. The reaction was started by the addition of 0.2 ml of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of butanol. Allowed to stand for 10 min and then centrifuged. The intensity of the chromogen in butanol layer was measured at 560 nm against butanol blank and the system devoid of enzyme as control.

One unit of enzyme activity was defined as the enzyme reaction which gave 50% inhibition of NBT reduction/minute and expressed as specific activity in units/g tissue.

3.13.4.13. Estimation of Catalase (Sinha, 1972)

Principle: Catalase causes rapid decomposition of hydrogen peroxide into water.

\[ \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2 \]

This method was based on the fact that dichromate in acetic acid reduced to chromic acetate when heated in the presence of \( \text{H}_2\text{O}_2 \) with the formation of perchloric acid as unstable intermediate. The chromic acetate thus produced was measured spectrophotometrically at 610 nm.

Reagents: Appendix XLV

Procedure: To 1.0 ml of the phosphate buffer, 0.1 ml of sample and 0.4 ml of 2 M hydrogen peroxide were added after 30, 60 and 90 seconds. 2 ml of dichromate acetic acid reagent was added to this mixture. To the “control tubes” the enzyme was added
after the addition of the acid reagent. The tubes were kept in a boiling water bath for 10 min and the colour developed was read at 610 nm.


**Principle:** The Glutathione Peroxidase was reacted with H$_2$O$_2$ in the presence of GSH for a specific time period then measured remaining GSH content at 412 nm.

\[
\begin{align*}
\text{GPx} \\
2\text{GSH} + \text{H}_2\text{O}_2 & \rightarrow \text{GSSH} + 2\text{H}_2\text{O}
\end{align*}
\]

**Reagents:** Appendix XLVI

**Procedure:** The reaction mixture containing 0.4 ml of sodium phosphate buffer, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, 0.1 ml of H$_2$O$_2$, 0.2 ml of enzyme homogenate, were made up to 2.0 ml with distilled water. The tubes were incubated at 37°C for 10 min. The reaction was terminated by the addition of 0.4 ml of TCA. To determine the residual glutathione contents, 2 ml of supernatant was collected by centrifugation and to this added 3 ml of disodium hydrogen phosphate and 0.5 ml of DTNB reagent. The yellow colour developed was read at 412 nm in a spectrophotometer. Standards were treated in a similar manner. A blank was treated with only disodium hydrogen phosphate and 1 ml of DTNB solution.

3.13.4.15. Estimation of Glutathione – S – Transferase (Habig et al., 1974)

**Principle:** Glutathione – S – transferase catalyses the reaction of 1 – chloro – 2, 4 dinitrobenze with the sulfhydryl group of glutathione.

\[
\begin{align*}
\text{CDNB} + \text{GSH} & \rightarrow \text{CDNB - S - glutathione}
\end{align*}
\]

The conjugate CDNB - glutathione, absorbs light at 340 nm and the activity of the enzyme can therefore be estimated by measuring the increase in the absorbance at 340 nm.

**Reagents:** Appendix XLVII

**Procedure:** The reaction mixture contained 1 ml of phosphate buffer (pH 6.5), 0.1 ml of 1- chloro - 2, 4 dinitrobenzene (CDNB) and 1.7 ml of double distilled water. After pre-
incubating the reaction mixture at 37°C for 5 minutes, the reaction was started by the addition of 0.1 ml of tissue homogenate and 0.1 ml of reduced glutathione as substrate. The absorbance was measured at 340 nm. Standard and blank (without sample) were treated in a similar manner.

3.13.4.16. Estimation of Lipid Peroxidation Indices (Nichans and Samuleson, 1968)

**Principle:** Malonaldehyde and other thiobarbituric Acid Reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid in the acidic condition to generate a pink coloured chromophore which was read at 535 nm.

**Reagents:** Appendix XLVIII

**Procedure:** The tissue homogenate was prepared in Tris - HCl buffer. 1 ml of the homogenate was combined with 2 ml of TCA- TBA - HCl reagent and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. The flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the sample was read at 535 nm against a blank. Acid was added to the 6 ml of the supernatant followed by 2 ml of 0.05 M 2- thiobarbituric acid and after 40 mins of incubation at 40°C, the absorbance of the samples were read at 443 nm against the reagent blank.


3.14.1. Treatment groups

The experimental rats were divided into 6 groups of 6 animals in each group, after two weeks of acclimatization period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>Healthy untreated rats</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>Diabetic rats</td>
</tr>
<tr>
<td>Glibenclamide Control</td>
<td>Drug Control (glibenclamide 600 µg/kg body weight)</td>
</tr>
<tr>
<td>Treatment I</td>
<td>Diabetic rats received 50% hydroethanolic extract of <em>Rubia</em></td>
</tr>
</tbody>
</table>
**cordifolia** Linn. (root) extract (400 mg/kg body weight) for 28 days.

Treatment II - Diabetic rats received 50% hydroethanolic extract of combined *Barleria cristata* L. stem extract and *Rubia cordifolia* Linn. (root) extract in the ratio 1:1 (200 + 200 mg/kg body weight) for 28 days.

Treatment III - Diabetic rats received 50% hydroethanolic extract of combined *Barleria cristata* L. stem and leaf extract in the ratio 1:1 (200 + 200 mg/kg body weight) for 28 days.

**PHASE IV**

3.15. Histological Architecture of Liver, Pancreas and Kidney Tissue of Control and Treatment group Rats (Luna, 1968)

Reagents: Appendix XLVIX

3.15.1. Tissue processing: The tissue was placed in 10% formalin (10% formalin in 0.9% sodium chloride) for one hour. They were left overnight in running water after securing the mouths of the vessels with cotton gauze. The tissues were dehydrated in ascending grades of isopropanol by immersing in 80% isopropanol overnight followed by 100% isopropanol for one hour. The dehydrated tissues were cleaned in two changes of xylene, one hour each. Then the tissues were impregnated with histology grade paraffin wax (melting point 58-60°C). The wax impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at 3 micron thickness. The sections were floated on a tissue floatation bath at 40°C and taken on a glass slide smeared with equal parts of egg albumin and glycerol. Wax in the sections were then melted in an incubator at 60°C and after 5 minutes, allowed to cool.

3.15.2. Tissue staining: The sections were de-paraffinised by immersing in xylene for 10 min in a staining jar. The de-paraffinised section were washed in 100% isopropanol and stained in Ehrlich’s hematoxylin for 8 min. After staining in hematoxylin, sections were
washed in tap water and dipped in acid alcohol (8.3% HCl in 70% alcohol) to remove excess stain. The sections were placed in a running tap water for 10 min. The sections were counter stained in 1% aqueous solution of eosin for 1 minute. The excess stain was washed in tap water and the sections were allowed to dry. Complete dehydration of the stained section was ensured by placing the section in the incubator at 60°C for 15 min. When the sections were cooled they were mounted using DPX mountent. The cell architecture in the liver was observed under high power objective in a microscope.

3.16. Antimicrobial Studies (Kavanagh, 1972; Collins and Lyne, 1970)

Antibiotic sensitivity test was performed by commonly used agar disc diffusion method which was designed to determine the smallest amount of the antibiotic needed to inhibit the growth of a micro organism. The resulting value was determined by measuring the diameter of growth inhibition (clear) zone surrounding the antibiotic disc.

Reagents: Appendix L

3.16.1. Preparation of Saponin Fraction from the plant material (Yan et al., 1996)

The powdered plant sample was defatted by petroleum ether for 3 hrs at 40°C. After filtering the petroleum ether, the sample was extracted with methanol for 3 hrs with mild heating. The combined methanol extract was concentrated and methanol extract of sample was obtained. To get crude saponin extract the sample was dissolved in methanol and acetone (1: 5 v/v) to precipitate the saponin. The precipitate was dried under vacuum, to get whitish amorphous powder named Crude Saponin Extract (CSE). Certain CSE was fractionated by applying to silica gel - 60 (230 - 400 mesh) column chromatography and eluted with chloroform – methanol – water (70: 30: 10). Five fractions were collected and the solvents were evaporated under reduced temperature. Fraction 1 was chosen based on detection of total saponin concentration for antimicrobial study.
3.16.2. Antibacterial Activity Determination

**Test organisms used:** *Staphylococcus aureus, Salmonella para typhi, E.Coli, Klebsiella pneumonia*, standard ciprofloxacin (5 µg/disc) was used.

**Preparation of inoculums:** The inoculums for the study were prepared in fresh Nutrient broth from preserved slant culture. The inoculums were standardized by adjusting the turbidity of the culture to that of McFlarland Standards. The turbidity of the culture was adjusted by the addition of sterile saline or broth.

**Preparation of sterile swabs:** Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving by packing the swabs in culture tubes, papers or tins etc.

**Sterilization of forceps:** Forceps were sterilized by dipping in alcohol and burning off the alcohol.

**Procedure:**

The standardized inoculum was inoculated in the plates prepared earlier by dipping a sterile loop in the inoculums, removing excess of inoculums by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60° after each application. Finally passed the swab round the edge of the agar surface. The inoculum were left to dry at room temperature with the lid closed.

Each petri dish was divided into 3 quadrants, in 2 quadrants extract discs such as A and B, (discs are soaked overnight in extract solution) and in one quadrant standard Ciprofloxacin (5mg) were placed, with the help of sterile forceps. Then petri dishes were placed in the refrigerator at 4°C or at room temperature for 1 hr for diffusion. Incubated at 37°C for 24 hrs. The zone of inhibition produced by plant extract and Ciprofloxacin antibiotic was observed. It was measured using a scale, divider or venirecalipers and recorded the average of two diameters of each zone of inhibition.
3.16.3. Antifungal Studies

Test organisms used: *Aspergillus niger, Aspergillus fumigates Aspergillus parasites and Candida albicans.*

Preparation of inoculum: The inoculum was prepared in fresh sabouraud’s broth from preserved slant culture. The inoculum was standardized by adjusting the turbidity of the culture to that of McFarland Standards. The turbidity was adjusted by the addition of sterile saline or broth.

Procedure: The standardized inoculum was inoculated in the plates prepared earlier by dipping a sterile loop in the inoculum, removing the excess of inoculums by pressing and rotating the swab firmly against the slides of the culture tube above the level of liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60° after each application. Finally passed the swab round the edge of the agar surface. The inoculum were allowed to dry at room temperature with the lid closed.

Each petri dish was divided into 3 quadrants; in 2 quadrants such as A an B plant extract (disc soaked overnight) and in one quadrant standard Clotrimazole were placed, with the help of sterile forceps. Then petri dishes were placed in the refrigerator at 4°C or at room temperature for 1 hour for diffusion. Then were incubated at 37°C for 24 – 48 hours. The zone of inhibition produced by plant extract, clotrimazole antibiotic were observed. It was measured using a scale, divider or venirecalipers and recorded the average of two diameters of each zone of inhibition.

3.17. Statistical Analysis: Data were reported as means ± SD by using the statistical package of social sciences (SPSS) version 10.0 for windows. Data from the replicates of each experiment conducted (n varying from 6 – 12) were analyzed using analysis of Variance (ANOVA) and the group means were compared by Duncan’s Multiple Range Test (DMRT). Values were considered statistically significant when P < 0.05 (Duncan, 1957) and thus the groups were compared as given in the table.